STUDIES ON DEXTRANSUCRASE

I. Formation of Riboflavinylglucoside in Dextran-producing Cultures of *Leuconostoc mesenteroides*

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INTRODUCTION

In 1941 Hehre first demonstrated that Berkefeld-filtered extracts from cultures of ***Leuconostoc mesenteroides*** were able to bring about the formation of dextran from sucrose, and the overall reaction was presented by equation (I) (Hehre, 1946).

$$\text{n. sucrose} \longrightarrow \underset{\text{dextran}}{(\text{D-glucose})_n} + \text{n. D-fructose} \qquad (\text{I})$$

The dextran produced by this enzymic synthesis has been ascertained by many investigators that it is composed largely of α-1, 6-glucopyranosidic linkage and some of α-1, 4- or α-1, 3-linkages. Dextransucrase, the enzyme which converts sucrose into dextran and approximately one molecular equivalent of fructose as was shown in equation (I), has been found in the cell-free culture fluids of various strains of ***Leuc. mesenteroides*** (Hehre, 1951; Tsuchiya et al. 1952) and of ***Streptococcus bovis*** (Bailey, 1959). Some of the properties of the isolated enzyme have been described (Hehre, 1946; Koepsell et al., 1953; Bailey et al., 1957; Neely, 1960), but any exact knowledge of the enzymic action on the mechanism of polymerization of sugar has not yet been acquired.

In the course of investigation on a variation in the ratio of carbon assimilated into polysaccharide to the total carbon metabolized in the presence of various inhibitors, such as antibiotics, metallic ions and vitamins under aerobic and anaerobic conditions, using both homopolysaccharide-producing bacteria (***Leuc. mesenteroides***) and heteropolysaccharide-producing bacteria (***Aerobacter aerogenes***), the authors found that ***Leuc. mesenteroides*** produced a large amount of riboflavinylglucoside in sucrose cultures containing riboflavin where the production of dextran was also taken place.

Riboflavinylglucoside (5′-D-riboflavin-D-glucopyranoside) was obtained at first by Whitby (1950, 1952, 1954) with acetone-dried powder of rat liver, and then by Katagiri, Tachibana and Yamada from maltose and riboflavin with the action of enzyme preparations from a mutant strain of ***Aspergillus oryzae*** (Katagiri and Tachibana, 1953; Tachibana and Katagiri, 1955), ***Escherichia coli*** (Katagiri, Imai and Yamada, 1954; Katagiri, Yamada and Imai, 1957) and from acetone-butanol-producing bacteria (Tachibana, 1955). It was suggested that riboflavin and riboflavinylglucoside played an important role on glucosyl carrier in the enzy-

mic synthesis of oligosaccharides and further the transglycosidation relating to riboflavin might be one of the synthetic processes of polysaccharides in microorganisms (Katagiri and Tachibana, 1956).

Therefore, the isolation of riboflavinylglucoside with high yield by *Leuc. mesenteroides* offers further investigations to ascertain whether its formation would be due to the activity of different transglucosidase which could reveal almost simultaneously to the synthesis of dextran, and also to ascertain whether the transglucosidation relating to riboflavin would lead the formation of glucose polymers of high molecular weight.

The present paper deals with the isolation of riboflavinylglucoside and dextran from cultures of *Leuc. mesenteroides.*

METHODS

Three strains of *Leuc. mesenteroides*, NRRL B-512, IFO. 3426 and L. 20, were used. Microdetermination of the flavin-compounds was carried out by paper-chromatography; after a portion of the fermentation liquor was centrifuged, ammonium sulfate was added to the supernatant solution, and then flavin-compounds in the solution were extracted with phenol by bubbling a stream of air. The phenol layer was spotted on sheets of filter paper (Toyo Filter Paper, No. 51) and they were developed by ascending method at first with the organic phase of n-butanol-acetic acid-water (4 : 1 : 5, by vol.) and then with the mixture of n-butanol-pyridine-water (6 : 4 : 3, by vol.). The spots on the paper corresponding to each of flavin-compounds were examined by ultraviolet lamp and then independently they were cut out. The pieces of paper thus obtained, were eluted with water and filtered. The amount of flavin-compound in the filtrate was determined by measuring the light absorption at 450 mμ in a Shimazu spectrophotometer, model QR.

RESULTS

1. *Isolation of Riboflavinylglucoside*

An aqueous medium, containing 10% sucrose, 0.5% KH_2PO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.03% $(NH_4)_2SO_4$, 0.05% polypeptone, 0.15% yeast extract and 100 mg% of riboflavin, was adjusted to pH 7.0 with sodium hydroxide and then sterilized. After inoculation with 4% of a 1—2 days' culture of *Leuc. mesenteroides*, NRRL B-512, grown in malt-extract at 25—28°C, the medium was incubated in the dark at 25—27°C for 3 days under stationary conditions. At the end of fermentation an equal volume of ethanol was added slowly with stirring to the final culture fluid and the mixture kept to stand overnight. A gummy precipitate formed was removed by decantation, and the supernatant solution was concentrated to a small volume under reduced pressure. The separated riboflavin

was filtered off. Ammonium sulfate was added to the filtrate, the mixture was shaken several times with a small amount of phenol, and the phenol extract was again shaken with one volume of water and ten volumes of ether in order to transfer all water-soluble flavin-compounds into the aqueous layer. The last operation was repeated twice, and the combined aqueous layer was supplied on paperchromatography. The flavin-compounds were separated from one another on a multiple chromatogram with two kinds of solvent systems; organic phase of n-butanol-acetic acid-water (4 : 1 : 5, by vol.) and n-butanol-pyridine-water (6 : 4 : 3, by vol.). The band on the paper corresponding to riboflavinylglucoside was cut out and eluted with distilled water by descending method. The eluate, after concentration, was resupplied on paperchromatography on which water saturated with isoamylalcohol was used as a developing solvent. And then the eluate of riboflavinylglucoside with water from paper sheets was evaporated to dryness at 40°C under reduced pressure, and the residue was dissolved in minimal amount of water. The insoluble impurities were removed by centrifugation. To the clear solution, sodium hydrosulfite was added, and the resulting precipitate of reduced riboflavinylglucoside was collected and then dissolved in a small amount of water. The solution thus obtained was subjected to column chromatography on Florisil. Riboflavinylglucoside was completely absorbed with Florisil and the resulting effluent revealed non-fluorescent. The column was washed with 5% acetic acid in order to separate sugars and other impurities, and again irrigated with 5% pyridine, whereupon riboflavinylglucoside was eluted. The effluent from the column was shaken in a separating funnel with chloroform to remove pyridine. The aqueous solution was evaporated under reduced pressure, and the residue was several times recrystallized from 80% aqueous ethanol until to get reddish yellow crystalline powder.

2. *Identification of Riboflavinylglucoside*

As it will be seen in Table 1, this crystalline powder revealed the same Rf values as riboflavinylglucoside previously obtained with acetone-dried powder of rat liver (Whitby, 1952), enzyme preparations of *Asp. oryzae* (Tachibana and Katagiri, 1955) and with *E. coli* (Katagiri, Imai and Yamada, 1954), when developed on paper with various solvent systems. It always gave only one spot on both multiple and two-dimentional paperchromatograms. The ratio of light absorption at 375 mμ to 450 mμ was 0.85. Its m. p. was 248—249°C on which the crystal turned brown in color and decomposed. It was readily soluble in water, sparingly soluble in ethanol, but insoluble in ether.

Its acid hydrolysis with N-hydrochloric acid at 100°C for 2.5 hours gave glucose and riboflavin, shown to be present in the molar ratio of 1 : 1 by colorimetric determination. Glucose was identified as a single reducing product of hydrolysis with aniline hydrogen phthalate (Partridge, 1949) on paperchromatograms developed severally with four different solvent systems; phenol-water (4 : 1, by vol.), n-butanol-acetic acid-water (4 : 1 : 2, by vol.), n-butanol-pyridine-water (6 : 4 : 3, by

TABLE 1
Rf values of riboflavinylglucoside isolated from the culture of *Leuc. mesenteroides*

Source of enzyme	Rat liver	*Asp. oryzae*	*E. coli*	*Leuc. mesenteroides*
Rf values #				
B. A. W.	0.20	0.21	0.20	0.21
P. W.	0.40	0.40	0.40	0.40
W. isoA.	0.50	0.48	0.51	0.50
B. F. W.	0.04	0.05	—	0.05
multiple paperchromatography				
B. P. W. —W. isoA.				one spot
P. B. W. —W. isoA.				one spot
two—dimentional paperchromatography				
W. isoA. —B. F. W.				one spot
P. W. —B. P. W.				one spot

Solvent systems:
B. A. W.; n—butanol-acetic acid—water (4 : 1 : 5, by vol.)
P. W.; $Na_2HPO_4 \cdot 12H_2O$—water (5 : 100)
W. isoA.; water, saturated with isoamylalcohol
B. F. W.; n—butanol—formic acid (80%)—water (77 : 10 : 13, by vol.)
B. P. W.; n—butanol—pyridine—water (6 : 4 : 3, by vol.)
B. F. W.; n—butanol—formic acid (80%)—water (4 : 1 : 1, by vol.)

vol.) and ethylacetate-acetic acid-water (9 : 2 : 2, by vol.). Determination of glucose was carried out by the Anthrone method (Scott and Meivin, 1953). After acid hydrolysis under conditions revealing minimal destruction of fructose (5mg substrate in one m*l* of 0.25 N-hydrochloric acid was kept for 3 hours at 95°C), any bright-blue spot which will be given by free fructose or by acid labile fructose-containing oligosaccharides was never detected on the papers, with urea-phosphoric acid spray (Wise et al., 1955). (Figure 1). The phosphate test by the method of Allen (Nakamura, 1950) was negative.

Isolation and purification of riboflavin from the hydrolysate were carried out by the procedure of multiple paperchromatography, followed by the extraction with benzylalcohol, reduction with sodium hydrosulfite and recrystallization from ethanol. Riboflavin thus obtained was identical with an authentic specimen in absorption of spectra and Rf on paperchromatograms developed with the solvent systems; the upper phase of n-butanol-acetic acid-water (4 : 1 : 5, by vol.), n-butanol-pyridine-water (6 : 4 : 3, by vol.) or water saturated with isoamylalcohol.

From these data, the crystalline powder was identified as riboflavinylglucoside.

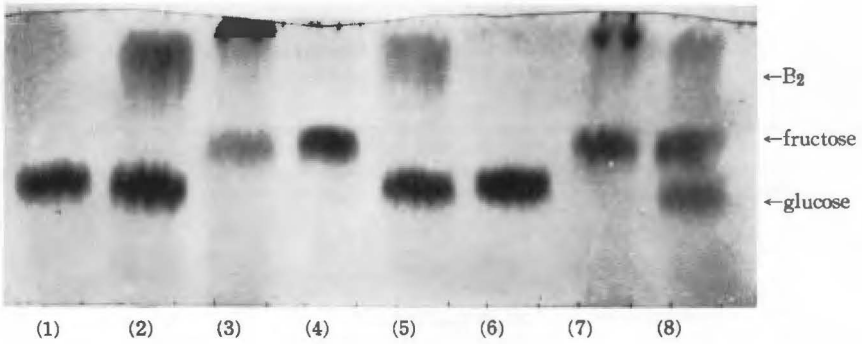

Fig. 1. Paperchromatograms of acid hydrolysate of riboflavinylglucoside isolated from the culture of ***Leuc. mesenteroides.***

Solvent system. phenol-water (4 : 1, by vol.)
Spray reagent. aniline hydrogenphthalate
(1). glucose
(2), (5). a acid hydrolysate of the sample treated with 0.5 N-hydrochloric acid for one hour at 95°C
(3), (7). fructose treated with acid in the same way as the sample
(4). fructose
(6). glucose treated with acid in the same way as the sample
(8). fructose
+
a acid hydrolysate of the sample treated with 0.5 N-hydrochloric acid for one hour at 95°C

3. *Production of Riboflavinylglucoside from Riboflavin and Sucrose in Growing Culture of Leuc. mesenteroides*

The fermentation experiments were carried out with a medium containing 10% sucrose, 0.5% KH_2PO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.03% $(NH_4)_2SO_4$, 0.05% polypeptone, 0.15% yeast extract and the requisite amount of riboflavin. The pH of the medium was adjusted to 7.0 with sodium hydroxide. The medium was dispensed in 50 m*l* quantities in 100 m*l* Erlenmeyer flasks or 200 m*l* shaking flasks, and then sterilized. Two m*l* of a 24-hours' culture of *Leuc. mesenteroides*, IFO. 3426 or L. 20 which was each grown in 10 m*l* of the seed medium at 25—28°C, were used as the inoculum. The composition of the seed medium was 4% sucrose, 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 2 mg% $FeSO_4 \cdot 7H_2O$, 2 mg% $MnSO_4$, 0.06% $(NH_4)_2SO_4$, 0.2% yeast extract and 0.05% polypeptone. After incubation at 27—30°C for 1—2 days under stationary or shaking conditions, the fermentation broths were centrifuged and the supernatant solutions were assayed chromatographically as was mentioned above. The results of the experiments are presented in Tables 2 and 3. As is shown in Table 2, *Leuc. mesenteroides* produced a large amount of riboflavinylglucoside (about 70% of riboflavin added) and a small quantity of riboflavin compounds of oligosaccharides with which lower values of Rf than that of riboflavinylglucoside were observed in the solvent system of n-butanol-pyridine-water (6 : 4 : 3, by vol.).

TABLE 2
Production of riboflavinylglucoside

Type of culture	Shaking	Stationary
Time of incubation (hr.)	21	21
B_2 present (mg/50m*l*)		
{ initial	20.0	20.0
{ final	5.6	3.5
B_2—glucoside produced (mg/50m*l*)	13.6	15.5
B_2—oligosaccharides produced (mg/50m*l*)	0.7	0.9

TABLE 3
Production of riboflavinylglucoside in sucrose medium supersaturated with riboflavin, by shaking culture for 2 days

Experiment	1	11
B_2 present (mg/50m*l*)		
{ initial	30.0	50.0
{ final	+	8.0
B_2—glucoside produced (mg/50m*l*)	23.0	34.7
B_2—oligosaccharides produced (mg/50m*l*)	6.5	6.9

Furthermore, it will be seen in Table 3 that such a remarkable production of riboflavinylglucoside took place after 2 days' incubation when 34.7 mg riboflavinylglucoside was obtained from 50 mg riboflavin in 50 m*l* of fermentation broth, in which the bacterium was cultivated in the presence of an excess of riboflavin.

4. *Isolation and Identification of Riboflavinylisomaltoside*

Isolation and purification of riboflavin-compounds of oligosaccharides were carried out in the similar way as described in the case of riboflavinylglucoside. The Rf values of these purified preparations were found to be consistent respectively with those of riboflavin compounds of oligosaccharides obtained by *E. coli* (Katagiri, Yamada and Imai, 1958) (Table 4). The substance of Rf 0.53 was hydrolysed

TABLE 4
Rf values of riboflavin-compounds

Organism	*E. coli*		*Leuc. mesenteroides*	
Solvent systems	W. isoA.	B. P. W. #	W. isoA.	B. P. W. #
B_2	0.40	0.43	0.40	0.43
B_2—glucoside	0.50	0.31	0.49	0.31
B_2—isomaltoside	0.53	0.22	0.53	0.21
B_2—dextrantrioside	0.57	0.16	0.57	0.15

B. P. W. #: n-butanol—pyridine—water (6 : 4 : 3, by vol.)

with 2N-hydrochloric acid at 100°C for 1.5 hours. Paperchromatography of a hydrolysate revealed the presence of glucose as the only reducing sugar together with riboflavin. About two moles of glucose against one mole of riboflavin were observed by the determination (Scott and Meivin, 1953) in the hydrolysate. Further, the paperchromatographic analysis of a partial hydrolysate of the substance with 0.2—0.5 N-hydrochloric acid for one hour at 100°C showed components of Rf values identical with glucose, riboflavin, riboflavinylglucoside and isomaltose.

From these experimental results the substance of Rf 0.53 was assumed to be riboflavinylisomaltoside.

5. *Isolation of Dextran*

For the isolation of dextran, three species of ***Leuc. mesenteroides***, NRRL B-512, IFO. 3426 and L. 20, were used. All the stock cultures were successively transfered bimonthly on bouillon-sucrose agar slants. To prepare the inocula, one loopful of stock culture was suspended in 10 m*l* of the seed medium containing sucrose (4%, w/v) and then incubated at 25—28°C for 2 days. This culture was then transfered to 100 m*l* of 10% sucrose medium employed in the experiment shown in Table 2, but without riboflavin, and incubated for 2 days. This was used as the inoculum of the fermentation studies on the isolation of dextran. The fermentation was carried out with 900 m*l* of 10% sucrose medium containing riboflavin in one liter Erlenmeyer flask after inoulation at 10 percent level and incubation at 25—28°C for 4 days under stationary conditions. At the end of dextran production, some cultures (NRRL B-512 and IFO. 3426) became remarkably viscous, and the other (L. 20) was not viscous but flocculent particles appeared in the solution. Two volumes of ethanol were then added to each culture. A white gummy material was precipitated. It was filtered, washed with alcohol and dried in reduced pressure. Each crude material by various strains appeared different solubility; readily water-soluble, less soluble and insoluble materials were obtained by NRRL B-512, IFO. 3426 and L. 20, respectively. This insoluble material was rendered soluble in water by dissolving into strong acid (Darker and Stacey, 1938) or alkali (Jeanes et al., 1954), centrifuged and poured the clear solution into two volumes of ethanol. Each crude material treated with acid or alkli was purified by several reprecipitations with ethanol. Results are presented in Table 5. Each purified preparation had no reducing power on Fehling's solution and gave no color reaction with iodine. Complete acid hydrolysate of the preparation with 1.5 N-sulphuric acid at 100°C for 2.5 hours was found to contain 92 to 101% reducing sugar calculated as glucose. Only glucose was detected as reducing sugar by paperchromatography with the hydrolysate. Qualitative tests for fructose, uronic acid and pentose were negative. Partial acid hydrolysate of each preparation with 0.1 N-hydrochloric acid for 3 hours at 95°C was shown to contain components corresponding to glucose and an apparently homologeous of oligosaccharides with aniline hydrogen phthalate (Partridge, 1949) or with urea-phosphoric acid spray (Wise et al., 1955). The first

TABLE 5
Analyses of gummy materials produced by three strains of *Leuc. mesenteroides* in liquid media containing riboflavin

strain	Method of purification	Glucose content (%)	Nitrogen content (%)	$[\alpha]_D^{25°}$ (c=1, in N—KOH)
NRRL B—512				
	treated with			
	HCl	101.3	0.01>	+198°
	NaOH	95.3	—	—
IFO. 3426				
	treated with			
	HCl	98.8	0.01>	+192°
	NaOH	99.2	—	+198°
L. 20				
	treated with			
	HCl	92.3	0.01>	+198°
Clinical dextran		101.8	—	+200°

Nitrogen and glucose were estimated by the method of micro-Kjeldahl and Bertrand, respectively.

two of these oligosaccharides were chromatographically identical with authentic specimens of isomaltose and isomaltotriose on papers developed with n-butanol-pyridine-water (6 : 4 : 3, by vol.) or phenol-water (4 : 1, by vol.).

Thus the above these mucilages were confirmed to be dextran.

SUMMARY

During investigations on the synthesis of glucose polymers by microorganisms, it was found that strains of ***Leuconostoc mesenteroides*** produced a large amount of riboflavinylglucoside from sucrose in the presence of riboflavin in their growing cultures and under the same cultural conditions dextran was ascertained to be produced by the bacteria.

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